ISOLATION, STRUCTURE AND SYNTHESIS OF A TRICARBOXYLIC PORPHYRIN FROM THE HARDERIAN GLANDS OF THE RAT

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The Harderian gland is a compound tubulo-alveolar apocrine gland, located in each orbit, posterior to the eyeball. These glands were discovered in 1864 by the celebrated Swiss physician, Johann Jacob Harder, and they have been found in those animals possessing a *membrana nictitans* [1]; it is thought that the function of the secretion is to lubricate this "third eyelid". The first studies of the amorphous pigment in the Harderian glands were made by Derrien and Turchini in 1924 [2] using albino and piebald rats, and they identified this pigment as a porphyrin. The solubility and spectroscopic properties of this porphyrin were very similar to those of the porphyrin of egg-shells and the Kämmerer porphyrin, which were subsequently shown to be protoporphyrin-IX by Fischer [3]. Many later workers confirmed the presence of protoporphyrin in the glands, and much of the more recent work has been concerned with the possible relationship between porphyrin biosynthesis in the glands, and the susceptibility of the animals to cancer.

In 1954, one of us (G.Y.K.) showed that a tricarboxylic porphyrin is also present in the glands of normal rats. He subsequently showed that the glands are normally capable of synthesising porphyrin [4], and this was later confirmed by other workers, notably Davidheiser and Figge [5], and more recently by Tomio and Grinstein [6]. We have now isolated the pure compound which we name, *Harderoporphyrin*, and we have defined its structure as (Ia).

In the present work, the porphyrin esters obtained by the extraction of the glands of normal Wistar rats with methanol containing 5% v/v sulphuric acid have been separated into two fractions by column chromatography on magnesium oxide (Grade III) in chloroform. The main band, eluted first, was protoporphyrin-IX dimethyl ester, whilst the second band (the Harderoporphyrin) eluted with chloroform contaning 0.5% v/v methanol, was shown to be the trimethyl ester of a tricarboxylic porphyrin by paper and thin layer chromatography. The original sample of the Harderoporphyrin trimethyl ester obtained in this way had m.p. 151°C, and its acid number was 6.5, which is higher than that of protoporphyrin dimethyl ester (5.5). The electronic absorption spectrum in chloroform ($\lambda_{max.}$ 406, 508, 542, 576, 631 nm) was of the aetio type and closely resembled that of protoporphyrin dimethyl ester. On reduction with hydriodic acid in glacial acetic acid a slight shift to shorter wavelengths was observed ($\lambda_{max.}$ 402, 506, 536, 571, 627 nm in ether) and this shift is consistent with the presence of one vinyl group in the original porphyrin. The absence of aldehyde or keto groups in the Harderoporphyrin was confirmed by the lack of reactivity towards hydroxylamine, and the absence of hydroxylic substituents was demonstrated by its inertness to attempted acetylation and benzoylation.

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Further studies of the Harderoporphyrin were carried out on material obtained both from the glands of normal Wistar rats, and from rats bearing the RD/3 (Sheffield) sarcoma. The crude mixture of porphyrin methyl esters, extracted from the glands of 600 normal Wistar rats as described above, was chromatographed on thick layer plates (silica) in light petroleum (b.p. 60–80°)/acetone (4/1:v/v) to remove fatty material. All the porphyrin was then extracted from the plates with methylene chloride/methanol and hydrolysed with methanolic potash. The mixture of free acids obtained was separated into three components by counter current distribution (100 transfers)** between 1.3 M H_2SO_4 and methyl isobutyl ketone/benzene (1/1:v/v) [7], and each of the three fractions was then reconverted into the corresponding methyl ester with methanol containing 5% v/v sulphuric acid.

The first fraction (K = 6.49, max. at tube no. 88) was identified as protoporphyrin-IX, and the third fraction (K = 0.081, max. at tube no. 8) was tentatively identified as coproporphyrin-III.

The middle fraction (K = 0.50, max. at tube no. 33) appeared to be a tricarboxylic porphyrin from its position in the distribution ***, and was identical with the Harderoporphyrin obtained in the earlier studies. The mass spectrum of the methyl ester showed a parent ion at m/e 650, and fragment ions corresponding to the succesive loss of three $CH_2CH_2CO_2CH_3$ groups [8]. Furthermore the molecular weight and visible spectrum confirmed the earlier work in showing that the Harderoporphyrin contained one vinyl substituent; on the assumption that it was closely related to protoporphyrin-IX it was assigned structure (Ia) or (Ib).

Similar results were obtained with material extracted from the Harderian glands of 700 Wistar rats bearing the RD/3 (Sheffield) sarcoma, and the amounts of each porphyrin recovered from the glands are summarised in the table. During the course of this work Tomio and Grinstein [6] also showed that protoporphyrin-IX, coproporphyrin-III and a tricarboxylic porphyrin were present in Harderian glands by paper chromatographic experiments.

To distinguish between the two possibilities (Ia) and (Ib) we have now synthesised both porphyrins by our recently devised b-oxobilane route [9,10], from the pyrromethanes (IIa) and (IIb) and the α-free pyrromethane (III). Transformation of the acetoxyethyl side-chains into vinyl groups at the porphyrin stage was effected by the same method as we had previously used [10] for protoporphyrin-IX. The mono-vinyl porphyrins (Ia) and Ib) were obtained as their trimethyl esters, m.p. 179–181°C and 176–178°C respectively.

Table
Porphyrins obtained from rat harderian glands.

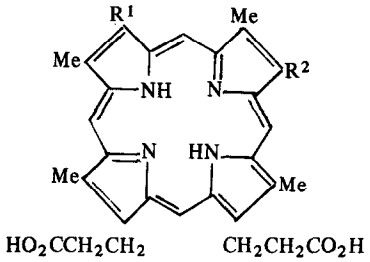
	700 Normal rats		600 rats bearing RD/3 Sarcoma	
	Yields [a] (mg)	Relative % [b]	Yields [a] (mg)	Relative % [b]
Protoporphyrin-IX	3.4	64	3.5	68
Harderoporphyrin	1.7	29	1.1	24
Coproporphyrin-III	0.4	9	0.6	8

[a] Crystalline material isolated.
[b] Relative yields estimated spectroscopically from counter current distribution. The total spectroscopic yield was about twice the amount finally obtained in crystalline form.

Mixed m.p. comparisons with the trimethyl ester of the natural material (m.p. 179°C) demonstrated that it was the 2-vinyl porphyrin (Ia). The mass spectrum of the synthetic 2-vinyl porphyrin was also rather more similar to that of the natural material than was that of the 4-vinyl isomer. However, a much more convincing confirmation of the structural assignment was provided by further counter current distribution experiments. A test mixture of the free acids, (Ia) and (Ib), derived from the two synthetic esters, separated partially after 600 transfers in the same system as before (K = 0.51 and 0.43 respectively), whereas a mixture of the Harderoporphyrin and the synthetic 2-vinylporphyrin tricarboxylic acid did not show any evidence of separation after a similar distribution.

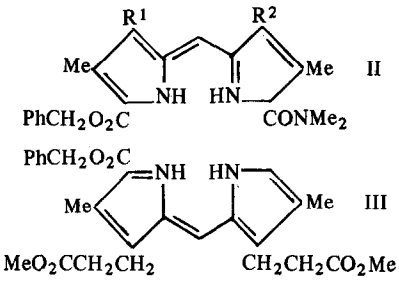
Harderoporphyrin is thus clearly shown to be 4,6,7-tri(2-carboxyethyl)-1,3,5,8-tetramethyl-2-vinylporphyrin (Ia). The relatively large amount found in the glands is of considerable interest in relation to the biogenesis of protoporphyrin and it seems likely that

** The temperature was in the range 18–23°C for the different runs and hance the partition coefficient varied slightly.
*** Model experiments were carried out with a synthetic tricarboxylic porphyrin.



I a) $R^1 = CH{=}CH_2$
$R^2 = CH_2CH_2CO_2H$

b) $R^1 = CH_2CH_2CO_2H$
$R^2 = CH{=}CH_2$



II a) $R^1 = CH_2CH_2OAc$
$R^2 = CH_2CH_2CO_2Me$

$R^1 = CH_2CH_2CO_2Me$
$R^2 = CH_2CH_2OAc$

the Harderian porphyrin is formed by dehydrogenation of an intermediate in the conversion of coproporphyrinogen-III to protoporphyrinogen-IX [11]. If this is so, modification of the 2-propionic acid (in coproporphyrinogen) precedes that of the 4-propionic acid in the biosynthetic sequence. The reason for the greater reactivity of the 2-propionate group may perhaps be deduced from the relative rates of enzymic decarboxylation of the coproporphyrinogen isomers [12]; types I and II are inert, whereas type IV decarboxylates at one tenth the rate of type III. If vicinal propionate groups are essential for binding of the enzyme, then type I cannot bind, and in type II there are two pairs of vicinal propionate groups but decarboxylation of any one propionate is likely to be hindered by interaction with its neighbour. In type IV both the 1- and 4-propionate groups are in the same relative orientation to the vicinal propionate groups (at positions 6 and 7) as is the 4-propionate group of type III. Therefore, as type III is known to be decarboxylated more rapidly than type IV, it is the 2-propionate which is degraded the faster. It is perhaps significant that in two porphyrins from other mammalian sources, pemptoporphyrin [10,13] ($I, R^1 = H, R^2 = CH{:}CH_2$) and chlorocruoroporphyrin [10,14] ($I, R^1 = CHO, R^2 = CH{:}CH_2$) the group in the 2-position has been even further degraded. It will be interesting to discover whether the porphyrin, recently isolated from meconium [15] and shown by mass spectrometry † to be a dehydrocoproporphyrin, has an acrylic acid side-chain also at the 2-position. The relatively large accumulation of free porphyrins in the Harderian gland (rather than haem) is presumably due to the lack of an iron-chelating enzyme.

† Mass spectrum determined at Liverpool with an A.E.I. MS9 spectrometer.

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